REMARKS

The newly added claims recite aspects of the invention and are fully supported by the specification. New independent claim 16 is similar to claim 1 and also recites a modification disclosed at page 3, lines 3-11. New independent claim 17 is drawn to human AR (the elected species), but not to human ERβ. The new claims do not require a new search and thus do not present an undue examination burden for the PTO, and they reduce issues of patentability. It is thus requested that these claims be entered and examined.

The requirement to incorporate by reference the amino acid sequence of SLIM-3 is improper. As applicants pointed out in the Reply of December 26, 2001, the sequence of SLIM-3 is readily available to the public and is not essential to practicing the invention. The sequence is not even recited in the claims. Nevertheless, in the interest of expediting prosecution, the sequence of the protein, as presented in the Magwick reference that is cited on page 1, line 13 of the instant specification, has been incorporated *verbatim* into the specification. See, *e.g.*, Figure 2 of Magwick. A copy of the Magwick reference is attached hereto for the convenience of the Examiner.

The enablement rejection is traversed. The Examiner's allegations concerning biologically active fragments are now moot. With respect to the foregoing amendments, note that no reasons have been given as to why claims 13-15 are allegedly not enabled.

As for the rejection under 35 USC 112, second paragraph, applicants disagree that the term "biologically active" is unclear, for at least the reasons presented in the Reply of December 26, 2001. Nevertheless, to expedite prosecution, the claims have been amended to clarify even

further the meaning of this term. The amendment does not narrow the scope of the claims.

In view of the preceding amendments and arguments, the application is believed to be in condition for allowance, which action is respectfully requested.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

On page 1, beginning at line 11, replace the following paragraph as follows:

The protein SLIM3 was first described by M.J. MORGAN and A.J.A. MADGWICK (1996) Biochem. Biophys., Res. Commun., Vol. 225, pp. 632-638. SLIM3 has 279 amino acids. M A E K F D C H Y C R D P

L Q G K K Y V Q K D G H H C C L K C F D

K F C A N T C V E C R K P I G A D S K E

V H Y K N R F W H D T C F R C A K C L Q

P L A N E T F C G Q G Q Q R S C A Q C T

T X E D F P K C K G C F K A I V A G D Q

N V E Y K G T V W H K D C F T C S N C K

Q V I G T G S F F P K G E D F Y C V T C

H E T K L A K H C V K C N K A I T S G G

I T Y Q D Q P W H A D C F V C V T C S K

K L A G Q R F T A V E D Q Y Y C V D C Y

K N F V A K K C A G C K N P I T G F G K

G S S V V A Y E G Q S W H D Y C F H C K

K C S V N L A N K R F V F H Q E Q V Y C

P D C A K K L

Please amend claims 1 and 13 as follows:

1. (Twice Amended) A method of identifying an ~~agents~~ agent that ~~regulate~~ regulates the transcriptional activating activity of human AR or ERβ, comprising:

contacting a cell expressing human androgen receptor (AR) or human estrogen receptor β (ERβ), and, human skeletal muscle LIM protein (SLIM)3~~, or biologically active fragments thereof,~~ with a test agent; and

determining whether said test agent regulates the transcriptional activating activity of human AR or human ERβ.

13. (Amended) A method of identifying an ~~agents~~ agent that ~~regulate~~ regulates the transcriptional activity of human AR or ERβ, comprising:

contacting a cell expressing human AR or human ERβ, and human SLIM, or biologically active polypeptides having at least 90% sequence identity thereto, with a test agent; and

determining whether said test agent regulates the transcriptional activating activity of human AR or ERβ.

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 225, 632–638 (1996)
ARTICLE NO. 1222

Slim Defines a Novel Family of LIM-Proteins Expressed in Skeletal Muscle

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Received July 11, 1996

We have assembled the complete protein sequence of the skeletal muscle LIM-protein SLIM by aligning overlapping cDNA sequences. These cDNA sequences were identified from our own sequencing and from BLASTn searches of non-redundant cDNA databases. The predicted SLIM protein sequence included four LIM-domains and a novel single zinc finger domain located in the N-terminal region. Similar sequences to SLIM were identified and termed SLIM2 and SLIM3. The SLIM3 cDNA sequence was identified subsequently as a partial sequence of the of the LIM-protein DRAL. The number and spacing of the LIM domains was common to all three protein sequences. The mRNA for each protein was detected in human masseter muscle RNA by Northern analysis. We suggest that these proteins belong to a novel family of LIM proteins that are expressed in human skeletal muscle. © 1996 Academic Press, Inc.

LIM-proteins are a class of protein that are defined by the possession of a highly conserved double zinc finger motif, $CysX_2CysX_{16\text{-}23}HisX_2CysX_2CysX_2CysX_{16\text{-}23}CysX_2Cys/His/Asp$, where the Cys and His residues co-ordinate the binding of two Zn^{2+} ions (1). The acronym LIM derives from three such proteins Lin-11, Isl-1 and Mec3 (2-4). The function of LIM-domains remains unclear, although a number of LIM-proteins have been associated with key points of development and differentiation (5) and it has been postulated that LIM-domains may provide a protein/protein binding interface (6). Four classes of LIM-proteins so far have been identified and are classified by (I) the presence of homeodomains, (II) the presence only of LIM-domains, (III) the presence of LIM-domains at the C-terminal or (IV) the presence of a diverse range of functional domains (5,7). A developmentally regulated LIM-protein, SLIM, is expressed in skeletal muscle but not in a variety of other tissues (8).

We report here the complete protein sequence for SLIM and the identification of two similar LIM-proteins termed SLIM2 and SLIM3. SLIM3 was found to be homologous with DRAL, a cDNA clone isolated from human skeletal muscle (9).

MATERIALS AND METHODS

Derivation of sequences. The plasmids pHSB10b12, pHSB20d02 and pHSBa2a04 were obtained from Genethon (France). The cDNA inserts of pHSB10b12 and pHSB20d02 were sequenced using the Sequenase 2.0 kit (US Biochemical Corporation, USA). The partial cDNA sequence for SLIM was used to identify overlapping and homologous cDNA sequences using the BLASTn search program of non-redundant databases located at the National Centre for Biotechnology Information. Such sequences were aligned using GeneWorks (Release 2.45 supplied by Intelligenetics). By a process of iteration the sequence was extended until no further overlapping sequences could be identified. CLUSTAL W (v1.5) was used to align the predicted protein sequences. Similarly, the predicted sequences for SLIM2 and SLIM3 were assembled from the following sequence data: accession numbers Z19368 (including the further sequencing of this clone), Z19178 and Z19179 for SLIM2; and accession numbers T39706, R57539, R57600, R57861 and T34559 for SLIM3.

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Abbreviations: RTPCR, reverse transcription polymerase chain reaction; SLIM, skeletal muscle LIM-protein; 3′-UTR, 3′ untranslated region; nt, nucleotide; bp, base pair.

0006-291X/96 $18.00

TABLE 1

p1	acgaattcGTGGCCAAGAAGTGTGCTGG	SLIM1	sense 731–750
p2	aagaattcTTGGCCAAGCATTGCGTGAA	SLIM1	sense 554–573
p3	tcgaattcGACTTCCCCAAGTGCAAGGG	SLIM1	sense 372–391
p4	cagaattcTGTGCCAACACCTGTGTGGA	SLIM1	sense 189–208
p5	atgaattcATGGCGGAGAAGTTTGACTG	SLIM1	sense 84–103
p6	ctctcgagCAGCTTTTTGGCACAGTCGG	SLIM1	antisense 902–922
p7	tggatccATCTGCCACCGCTGCCAGC	SLIM3	sense 3–22
p8	agaattcTGTGTGAGATCACAAGCAGC	SLIM3	antisense 482–501

The oligonucleotides were purchased from GeneSys UK (p1-p6) and Pharmacia UK (p7, p8). Every oligonucleotide sequence is printed in a 5′ to 3′ orientation. The upper case sequence corresponds to the target sequence while the lower case sequence describes an additional restriction enzyme cut-site.

cDNA preparation, oligonucleotides, and PCR. The cDNA used to detect SLIM was isolated as an EcoR1 fragment from our plasmid pSKslim (U60118). The cDNA used to detect SLIM2 was isolated as an EcoR1 fragment from the plasmid pHS20d02. The cDNA used to detect SLIM3/DRAL was isolated using RTPCR. The oligonucleotides used for PCR are listed in Table 1. Single stranded cDNA was made using 5μg of human masseter total RNA (see below) primed with oligo-dT and other reagents from a kit supplied by Stratagene (UK). A PCR reaction mixture of 50μl, covered with 35μl of mineral oil (Sigma, UK), contained reaction buffer (GeneSys, UK), 250μM dNTPs (Sigma, UK), 1.5mM $MgCl_2$, 1pmol of each primer (p7 and p8) and 1μl of template cDNA. 1U of Taq polymerase (Genesys, UK) in 5μl of reaction buffer was added after an initial 2 minute incubation at 94°C and prior to thermal cycling. The reaction mix was incubated over 35 cycles of 30 seconds duration at 94°C, 55°C and 72°C sequentially, with a final incubation at 72°C for 270 seconds. The PCR products were size separated using agarose gel electrophoresis and the product corresponding to 513bp was excised from the gel and purified. A similar procedure was used in the analysis of SLIM mRNA with combinations of sense primers p1 to p5 and antisense primer p6. In additional PCR reactions, approximately 1ng of the plasmid pHSBa2a04 was substituted for cDNA to demonstrate the continuous nature of this SLIM clone.

RNA isolation and Northern blot analysis. Total RNA was isolated from a masseter autopsy sample using a guanidium isothiocyanate/phenol extraction procedure (10). Total RNA was quantified by absorbance spectrophotometry at 260nm. Dilutions of 1, 2, 4 and 8μg of RNA were size separated on a formaldehyde denaturing agarose gel and transferred by capillary action onto nylon membrane (Hybond N, Amersham International plc, UK). Ethidium bromide staining of the RNA permitted the visualisation of the bound RNA with exposure to UV light. Hybridisation of [^{32}P]-random-primer-labelled cDNA probe(s) (Multiprime kit, Amersham International plc, UK) was carried out over two hours at 68°C using "Quikhyb" solution (Stratagene, UK). This included a high stringency removal of unbound probe by washing the membrane in 0.2×SSC containing 0.1%SDS for 30 minutes at 65°C. Hybridised cDNA was detected by autoradiographic exposure over a period of four days.

GenBank. The sequences reported here have been submitted to the GenBank/EMBL data base and given accession numbers U60115 (SLIM1), U60116 (SLIM2) and U60117 (SLIM3).

RESULTS AND DISCUSSION

We have described previously the partial sequence of SLIM derived from clone B10b12 (8). Here we have extended the sequence of SLIM by combining physical sequencing of clones B10b12 and Ba2a04 with the alignment of overlapping sequences recovered from the database (Fig. 1A). The majority of the sequences were derived by others from a single skeletal muscle cDNA library (#936215, Stratagene). The aligned sequence was assembled from 36 overlapping cDNAs and predicted an mRNA with a length of 2254nt. Differences were observed between overlapping sequences and were assumed to arise as a consequence of sequencing errors. At points of sequence discrepancy, the most prevalent base from the aligned sequences was selected for the predicted cDNA sequence. Together, clones B10b12, Ba2a04 and Cztg10 spanned the sequence length as shown in Fig. 1A. The complete cDNA included a poly-adenylation site (AATAA) at 2235nt and a poly-A tail at 2255nt (Fig. 1A and Fig. 2). The largest open reading frame in the direction of the poly-A tail spanned nucleotides 84-923 and encompassed the partial cDNA sequence of pSKslim (Fig. 1A).

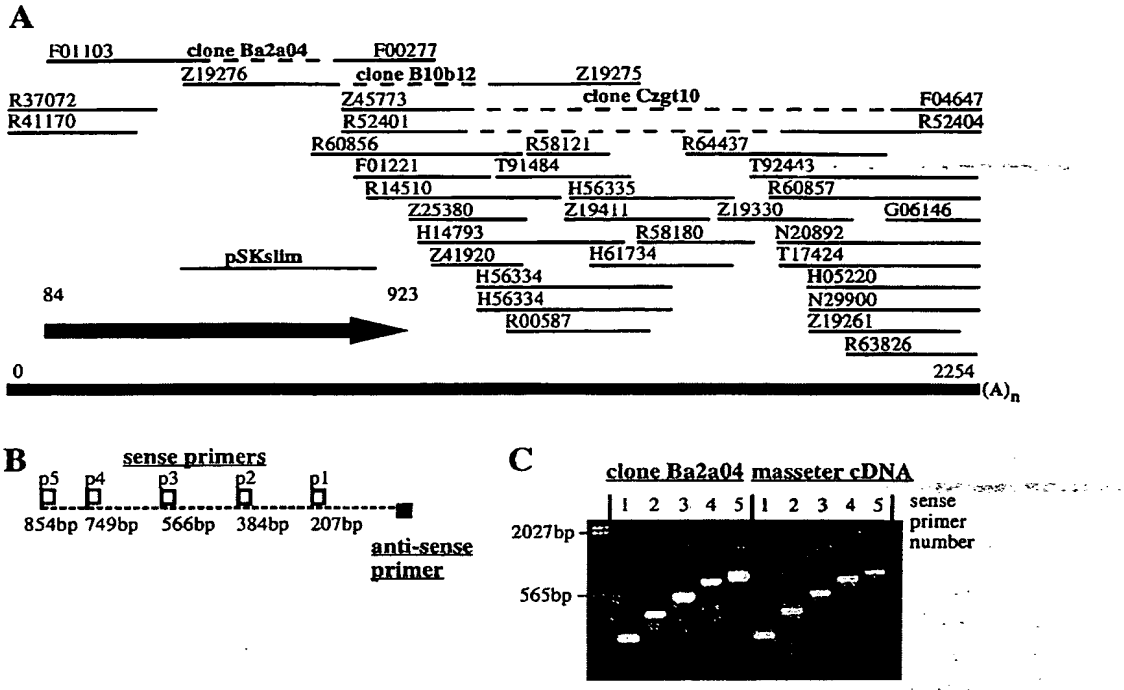

FIG. 1. (A) The predicted complete nucleotide and protein sequence for SLIM(1) where the alignment of nucleotide sequences (thin black lines) used to predict the SLIM1 mRNA sequence (thick black line) are shown. The labels refer to accession numbers. The dashed line represents an unknown sequence between two ends of a partially sequenced clone. The coding region is represented by the arrow pointing towards the C-terminal end and is aligned to the nucleotide sequence. (B) The orientation of PCR oligonucleotides to the predicted coding region of SLIM1. Open boxes represent the sense oligonucleotides p1 to p5, the filled box represents the antisense oligonucleotide (p6). The number of base pairs (bp) between every sense oligonucleotide and the antisense oligonucleotide is shown. (C) An ethidium bromide stained gel confirmed that the size-separated PCR products spanned the SLIM(1) coding region. The approximate sizes are indicated by the λ *Hind*III markers. The lane numbers refer to the sense oligonucleotide used in the PCR reaction. The clone Ba2a04 and masseter cDNA were used as target templates in the PCR reactions.

Comparative PCR analysis of masseter cDNA with cDNA clone Ba2a04 demonstrated that the corresponding mRNA sequence was present in the human muscle sample as shown in Figs. 1B and 1C; this confirmed that the predicted reading frame was a continuous sequence. The protein product had a predicted protein mass of 31.7kD and contained four LIM-domains. There was an uncharacterised single zinc-finger at the N-terminal end (amino residues 7-31) of the form $CX_2CX_{17}CX_2C$ (similar to a GATA-1 zinc finger or one half of a LIM-domain) as shown in Fig. 2. Every LIM-domain, including the single zinc finger, was separated by eight residues.

Two additional sequences were identified by their partial homology with the nucleotide sequence of SLIM. These sequences were named SLIM2 and SLIM3. SLIM2 was identified by combining extended physical sequencing of the clone B20d02 with the alignment of overlapping sequences recovered from clone B02c09 (Fig. 3A). Both clones had been isolated from the Stratagene skeletal muscle cDNA library. The predicted SLIM2 cDNA sequence contained 1420 nucleotides and included a poly-adenylation site 13 nucleotides from the poly-A tail (not shown). The predicted protein sequence included four LIM-domains and a stop codon. SLIM3 was assembled from five overlapping clone sequences. Although none of the clones were derived from a skeletal muscle library, three clones were derived from a fetal cardiac muscle

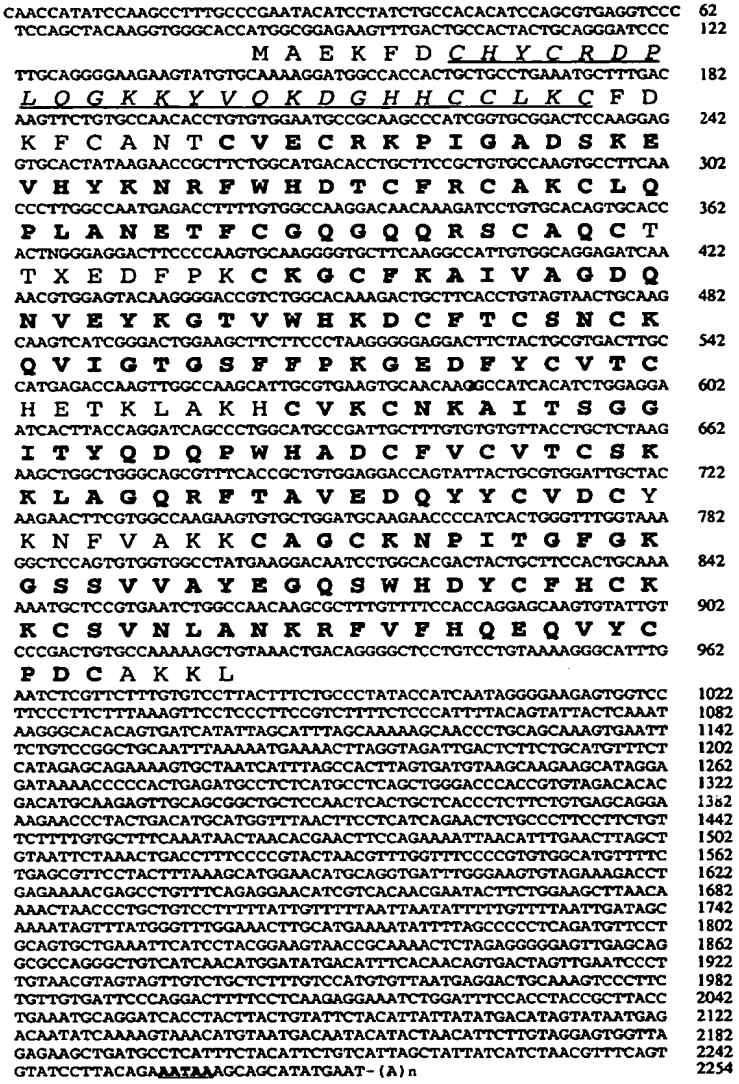

FIG. 2. The predicted cDNA sequence of 2254 nucleotides and the complete protein sequence of SLIM1. The predicted open reading frame spans amino residues 84-923 and is represented by the larger case letters. The amino acid sequences shown in bold correspond to the LIM-domain motifs. The uncharacterised zinc-finger motif is shown in underlined italics and the poly-adenylation nucleotide sequence in bold and underlined characters.

cDNA library (11). The SLIM3 cDNA sequence aligned to the coding region of SLIM and the predicted protein sequence included at least two LIM-domains and a stop codon (Fig. 3B). SLIM3 was shown to be homologous with the LIM-protein DRAL (9). The DRAL clone was isolated from a human neonatal skeletal muscle cDNA library and contained the complete nucleotide sequence. As with the two protein sequences described above, the DRAL protein sequence included four LIM-domains and an uncharacterised zinc finger domain in the N-terminal region. As a consequence of the identification of a family of similar protein sequences, we propose to rename SLIM as SLIM1.

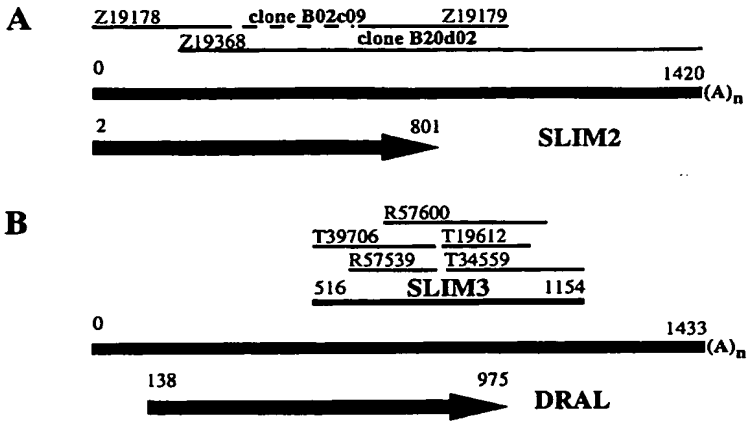

FIG. 3. The predicted nucleotide and protein sequences for SLIM2 (A) and SLIM3 (B). The alignment of SLIM 3 to DRAL is shown also. The notation is identical to that in Fig. 1A.

The CLUSTAL comparison of SLIM1, SLIM2 and DRAL aligned the amino acid sequences at LIM-domain cysteine and histidine motifs (Fig. 4). The length of the finger motifs and the length of the amino acid sequences flanking the LIM-domains were highly conserved. However

```
SLIM1   MAEKFDCHYCRDPLQGKKYVQKDGHHCCLKCFDKFCANTCVECRKPIGADSKEVHYKNRF
SLIM2           GRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
DRAL    MTERFDCHHCNESLFGKKYILREESPYCVVCFETLFANTCEECGKPIGCDCKDLSYKDRH
                    *.**.  .    *. *..   **** **  . ** *.... *  *
             [uncharacterised Zn finger]       [LIM1-

SLIM1   WHDTCFRCAKCLQPLANETFCGQGQQRSCAQCTTXEDFPKCKGCFKAIVAGDQNVEYKGT
SLIM2   FHRGCFRCCRCQRSLADEPFTRQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
DRAL    WHEACFHCSQCRNSLVDKPFAAKEDQLLCTDCYSNEYSSKCQECGKKTIMPGTRKMEYKGS
         *. **.*..*   *    *. .  .  * *  .     .*  *  ... * . .** *
                                  -LIM1]       [LIM2-

SLIM1   VWHKDQFTGSNCKQVIGTGSFFPKGEDFYCVTCHETKLAKHCVKCNKAITSGGITYQDQP
SLIM2   TWHEHCFLCIGCEQPLGSRPFVPDKGAHYCVPCYENNFAPRCARCTKTLTQGGLTYRDLP
DRAL    SWHETCFICHRCQQPIGTKSFIPKDNQNFCVPCYEKQHAMQCVQCKKPITTGGVTYREQP
         **  ** *  * * .*.  * *     .** * *   * .* .* * .* **.**.. *
                                  -LIM2]       [LIM3

SLIM1   WHADCFVCVTCSKKLAGQRFTAVEDQYYCVDCYKNFVAKKCAGCKNPITGFGKGSSVVAY
SLIM2   WHPKCLVCTGCQTPWQGTTSPPGMKNPYCVACFGKPLHSNCYSCNRPTLMTRWKQLLCSF
DRAL    WHKECFVCTACRKQLSGQRFTARDDFAYCLNCFCDLYAKKCAGCTNPISGLG-GTKYISF
        **  * **  *      *           **. *.        *  *  *          ..
                                  -LIM3]       [LIM4-

SLIM1   EGQSWHDYCFHCKKCSVNLANKRFVFHQEQVYCPDCAKKL-
SLIM2   ENRHWHQNCFTCDRCSNSLVGQGFVPDGDQVLCQGCIQAGP
DRAL    EERQWHNDCFNCKKCSLSLVGRGFLTERDDILCPDCGKDI-
        * . **  ** * .**  * .. *.   . . *  * .
                                  -LIM4]
```

FIG. 4. CLUSTAL W (v1.5) multiple sequence alignment of the predicted protein sequences of SLIM1, SLIM2 and DRAL. Homology is marked with an asterisk. Similar amino residues are marked with a period. The LIM-domains and the uncharacterised zinc-finger domain are denoted by square brackets. The shaded box highlights the cysteine-rich LIM motifs.

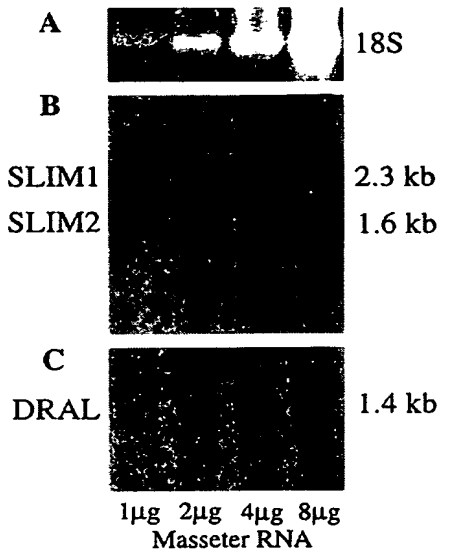

FIG. 5. The expression of the LIM-family mRNAs in human masseter muscle are shown. The ethidium bromide stained gel of 18S rRNA (A) and the corresponding Northern blot autoradiographs for SLIM1 and SLIM2 (B) and DRAL(C).

there was one less amino residue in the first LIM4 finger of DRAL and there was an additional amino residue at the C-terminal end of SLIM2. SLIM2 may contain the uncharacterised single zinc-finger at the N-terminal although sequencing of the 5′-end is incomplete (Fig. 4). When the amino acid sequences were compared, there was 47% identity between SLIM1 and DRAL, 45% identity between DRAL and SLIM2 and 36% identity between SLIM1 and SLIM2.

All three LIM-proteins were detected in human masseter muscle using Northern analysis. The signal intensities of SLIM1 and SLIM2 were similar (Fig. 5B). However there was a much lower signal intensity with DRAL (Fig. 5C) despite the probe being derived from masseter cDNA by PCR (not shown). Northern analysis indicated that the size (2.3kb) of SLIM1 mRNA from the human masseter was similar to that seen in other muscles and species (8). As predicted from the sequence data, SLIM2 and DRAL were of higher mobility and were calculated to be 1.6kb and 1.4kb in length respectively.

The coding regions of SLIM1, SLIM2 and DRAL are similar in length but the 3′ untranslated regions vary considerably. It is possible that the 3′-UTRs are involved in the regulation of SLIM-family gene expression. Whether the different family members have similar or different functions remains unclear. All the family members are expressed in skeletal muscle. SLIM1 has been shown to be elevated during postnatal skeletal muscle growth and may have an involvement in muscle development/hypertrophy (8). The presence of a zinc finger that is structurally similar to that found in GATA-1 suggests that the SLIM family members have the potential to bind DNA. Thus one may speculate that these proteins are involved in DNA/protein/protein interactions during muscle development and remodelling.

ACKNOWLEDGMENTS

We thank Dr. John Coadwell (Babraham Institute, Cambridge, UK) for his helpful advice on non-redundant sequencing and Dr. Marc Woodland (Imperial College, UK) for providing a DNA sequencing service.

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